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WINTER SURVIVAL OF BLOOD-FED AND NONBLOOD-FED *CULEX PIPPIENS* L.*

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Abstract. Comparisons were made between groups of *Culex pipiens* L. with different physiologic histories to test their ability to successfully overwinter under field conditions. On 14 December 1978, each group of mosquitoes was marked with a distinctive fluorescent dust and released inside an abandoned ammunition bunker at Fort Washington, Maryland. To insure that dead mosquitoes could be dissected and information obtained on their ovarian development, a sample of females from each group was also released into a plexiglass cage that was attached to the inside wall of the room. The physiologic histories of each group of mosquitoes were as follows: (a) "wild caught," those which had entered the bunker prior to the release date, (b) "lab-reared diapausing nonblood-fed," (c) "lab-reared diapausing blood-fed nongravid," (d) "lab-reared diapausing blood-fed gravid," (e) "lab-reared nondiapausing nonblood-fed," and (f) "lab-reared nondiapausing blood-fed." By 8 March 1979, all of the lab-reared nondiapausing groups, of mosquitoes released in the room had died, whereas 15.7, 22.4 and 24.7% were recovered from the "lab-reared diapausing nonblood-fed," "lab-reared diapausing blood-fed" (gravid and nongravid) and "wild caught" mosquitoes, respectively. For the mosquitoes in the cage, only 0, 2.1 and 7.0% of the "lab-reared nondiapausing blood-fed," "lab-reared nondiapausing nonblood-fed" and "lab-reared diapausing blood-fed gravid," respectively, survived. This compared to 45.4, 56.8 and 58.0%, respectively, for the "lab-reared diapausing nonblood-fed," "lab-reared diapausing blood-fed nongravid" and the "wild caught" groups. These data provide evidence to support the theory that a significant number of diapausing *Cx. pipiens* which have taken a prehibernation (possibly viremic) blood meal do not develop eggs and can survive the winter at rates comparable to diapausing nonblood-fed mosquitoes.

In temperate regions of its distribution the mosquito *Culex pipiens*, one of the vectors of St. Louis encephalitis (SLE) virus, overwinters as an inseminated adult female in a quiescent state, termed diapause. Diapause has been defined as a "phys-

iological state of suspended activity or arrested development that facilitates survival through a period of unfavorable conditions, but is initiated before the onset of these condition."¹ Diapause is induced in late-stage larvae and pupae in *Cx. pipiens*, by a short photophase in combination with cool temperatures. Induction of diapause by these latter factors is dependent on latitude; for example, in Boston, Mass., area day lengths of less than 12 hours and an average temperature less than 18°C provide a threshold for stimulation of diapause in *Cx. pipiens*.² Onset of diapause in these females has been shown to include: (a) reduction in blood-feeding drive, (b) hypertrophy of the fat body, and (c) lack of ovarian development following a blood meal (follicles remain in pre-resting stage NO_x-N of Kawai³).^{2,4} Spielman⁵ and Meola and Petralia⁶ demonstrated that blood-feeding drive and ovarian follicle size, respectively, in females preconditioned for diapause are influenced by natural or synthetic juvenile hormone (JH). Allatectomized females of *Cx. pipiens* show

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reduced blood-feeding drive and ovarian development similar to diapausing females when reared under nondiapausing conditions. Also, Spielman stated that hibernation (diapause) is disrupted,⁵ i.e., ovarian follicles develop, when diapause-conditioned females have been topically treated with or fed synthetic JH. These data suggest that diapause is controlled by the titer of JH present in the female, which has been secreted by the corpora allata. Likewise, we believe it may be the titer of JH which determines whether a diapausing female will develop eggs following a blood meal. The environmental factor or combination of factors necessary to terminate diapause is (are) not known; however it appears that diapause in the adults may be broken by warm temperatures over a period of a few days or possibly by long photophases.⁷

During the summer and fall in the east-central region of the U.S., *Cx. pipiens* is the primary vector of SLE virus. One of the most pursued questions regarding the ecology of SLE virus is the mechanism by which this virus survives the winter in this temperate region. Several hypotheses have been suggested^{8,9} and recently reviewed^{10,11} as potential mechanisms for the overwintering of arboviruses. Considerable evidence has been accumulated to support the role of transovarial transmission with members of the California group of arboviruses as a means for these viruses to survive adverse conditions. Recent laboratory evidence suggests that transovarial transmission of SLE virus to *Cx. pipiens* may be an important factor in infecting females entering diapause in the fall and infecting their progeny in the spring.¹² In this latter study, females maintained at 18°C transovarially transmitted virus at a higher rate than those maintained at 25°C. Another hypothesis for which there is supporting evidence is that the females become infected prior to diapause from a viremic blood meal.⁷ It is possible that both of these factors play a role in SLE virus transmission as illustrated in the hypothetical model of Eldridge.¹¹

Previous studies of prehibernation blood feeding have indicated that the rate of feeding in populations of *Cx. pipiens* females, preconditioned for diapause, is greatly reduced. Many investigators believe that this frequency is reduced to such a low level as to discount the possibility of an epidemiologically significant proportion of females becoming SLE-infected in the fall and then serving as overwintering reservoirs of this virus. How-

ever, Eldridge and Bailey demonstrated in laboratory studies that a significant proportion of females (18% of those exposed to 25°C for 72–84 hours), having been conditioned with short-day photoperiods (L:D 9:15) and cool temperatures (15°C), take blood meals when warmed to 25°C that do not result in the maturation of egg follicles.⁷ They state that in nature short warming periods are common during the months of September and October when short-day photoperiods exist. During these warm periods females may take an infected blood meal, remain nulliparous and then serve as an overwintering reservoir of SLE virus. Unfortunately, the only isolations of SLE virus from a potential vertebrate reservoir in the late fall and winter have been from bats collected in southeastern Texas;¹³ however, transmission of SLE virus during late fall in colder regions of North America is poorly understood.¹⁰ Evidence supporting a mosquito reservoir hypothesis has been the isolation of two strains of SLE virus from diapausing females of *Cx. pipiens* during the 1977–78 winter,¹⁴ and four more strains of SLE from this species during the winter of 1978–79.¹⁵ These females had survived the coldest part of the winter; therefore, it is believed that they would have survived until spring and could have reintroduced the virus into the vertebrate population or vertically transmitted the virus to their offspring.

The objective of this study was to test the hypothesis that a significant proportion of diapausing females of this species remain nulliparous after taking a blood meal and are capable of surviving the winter when sheltered in a hibernaculum. Inherent in this hypothesis is the assumption that if a female takes a blood meal and becomes gravid, she will not survive the winter. Also, females not preconditioned for diapause, whether blood-fed or nonblood-fed, will likewise be incapable of surviving the winter.

MATERIALS AND METHODS

Physiologic histories of mosquitoes tested

To test the above hypothesis under field conditions, overwintering survival comparisons were made between groups of females of *Cx. pipiens* with the following physiologic histories: Set I consisting of (a) "Wild caught," those females from the natural population which had entered the hibernaculum prior to the release date; (b) "Lab-reared diapausing nonblood-fed," and (c) "Lab-

reared diapausing blood-fed" (nongravid) and Set II, (d) "Lab-reared diapausing blood-fed (gravid)," (e) "Lab-reared nondiapausing nonblood-fed," and (f) "Lab-reared nondiapausing blood-fed."

Preconditioning of mosquitoes

The mosquitoes in groups b-f were descendents of a colony established in 1975 from diapausing females collected at Fort Mott, Salem Co., N.J. Larvae for the diapausing groups b, c and d were reared in climatic chambers at a temperature of 25°C, daily photoperiod of L:D 9:15 hours and relative humidity (RH) of 80-90%. When the first pupae appeared, the temperature was lowered to 15°C with no change in L:D and RH. When the youngest adults of groups c and d were 10 days old and the oldest 18 days old the mosquitoes were transferred to another climatic chamber at 25°C with the same photoperiod and RH. After 24 hours at 25°C, these mosquitoes (groups c and d) were offered 1- to 2-day old chicks for blood meals. Each morning for 3 successive days, all fully engorged females were removed and placed back into the 15°C environment. These mosquitoes were held for an additional 15-17 days under diapausing conditions to insure that the blood meal had been digested. The adults of group b were held for the same number of days in the 15°C environment and fed on a 10% sucrose solution before release.

For the nondiapausing groups e and f, the larvae and adults were reared in the same type of climatic chamber at 25°C, 80-90% RH and a long-day photoperiod of L:D 15:9 hours. When the adults of group f ranged from 5-9 days old, they were given blood meals on 1- to 2-day-old chicks and held for 8 days to allow for complete digestion of the meal. The nondiapausing, nonblood-fed group was held in the laboratory as adults from 8-11 days and fed on a 10% sucrose solution.

Marking of mosquitoes released inside Decator bunker

On 14 December 1978, each group of mosquitoes was marked with a distinctive fluorescent dust (Hercules Inc., Wilmington, NJ) and released inside a room of an abandoned ammunition bunker at Fort Washington, Md. Fluorescent dusts have been shown to have no significant effect on longevity of *Culex tarsalis*.¹⁶

Cage and room inside bunker

To insure that dead mosquitoes could be dissected and information obtained on their ovarian development, approximately half of the females from each physiologic group were released into a plexiglass cage attached 1 m from the floor to an inside wall of the room. The cage measured 75 × 75 × 50 cm and was enclosed on all sides except the back; the moist wall of the room served as a back for the cage and as a resting surface for the enclosed mosquitoes. A net-covered port on the front of the cage allowed easy access for removal of dead mosquitoes. Nylon netting covered the door to the room to minimize emigration and immigration of mosquitoes throughout the study period. Temperature and RH were monitored by a recording hygrothermograph in the room. Approximately twice a week the numbers of marked mosquitoes in each group in the room and in the cage were counted using a portable black light. For the room, the numbers of mosquitoes counted twice within a period were averaged, often resulting in a mixed number; for example, for the wild-caught group on day 39, the count at the beginning of the period was 49 and at the end of the period 48 (average 48.5). This was necessary because for the mosquitoes in the room it was impossible to be certain that all the mosquitoes were found on each occasion. In fact in some instances, as in the lab-reared diapausing nonblood-fed group on day 53, the average number actually increased indicating that during the previous period not all of the mosquitoes were found. This error in counting was not a factor inside the cage.

RESULTS

Overwintering survival of mosquitoes

The survival rates are given for every time period for the groups of females inside the room (Table 1) and for those in the cage (Table 2). Within the 1st week after release the numbers of marked mosquitoes free in the room had decreased to between 20-45% of their original numbers (Fig. 1). After the initial sharp decline, the numbers of mosquitoes in each group continued to decrease throughout the sampling period but at a slower rate. The mortality in the nondiapausing groups was greater than in the diapausing and wild groups so that the curves diverged. At the termination of the experiment (8 March) all of the nondiapausing

TABLE 1

Survival of *Cx. pipiens* in room of Decator Bunker at Fort Washington, Md, from 14 December 1978 to 8 March 1979

Accumulative time in days	Lab-reared									
	Wild-caught		Diapausing				Nondiapausing			
			Nonblood-fed		Blood-fed		Nonblood-fed		Blood-fed	
	No.	% survival	No.	% survival	No.	% survival	No.	% survival	No.	% survival
0*	170	100	178	100	161	100	100	100	96	100
4	100.5	59	128.5	72.2	127.5	79.2	70	70	53.5	55.7
11	67	39.4	124	69.7	115	71.4	67	67	38	39.6
18	58	34.1	86.5	48.6	109	67.7	35.5	35.5	14.5	15.1
25	50	29.4	65.3	36.7	78.3	48.6	25	25	22	22.9
32	50	29.4	59.5	33.4	73	45.3	19.5	19.5	15	15.6
39	48.5	28.5	48	27.0	69	42.9	20	20	18	18.8
46	49	28.8	38.5	21.6	57	35.4	8	8	9	9.4
53	45	26.5	43.5	24.4	57	35.4	11.5	11.5	11.5	12.0
60	43.5	25.6	36	20.2	53.5	33.2	5.5	5.5	6	6.2
74	39	22.9	29	16.3	47	29.2	2	2	2	2.1
84†	42	24.7	28	15.7	36	22.4	0	0	0	0

* 14 December 1978.

† 8 March 1979.

mosquitoes had died, whereas 15.7–24.7% of the diapausing and wild-caught mosquitoes remained alive.

For the mosquitoes released inside the cage, the survival curves of the groups in Set II strongly diverged from the curves of the groups in Set I (Fig. 2). Between 0–7.0% of the females in Set II were alive at the termination of the experiment versus 45.4–58.0% of those of Set I. A chi-square procedure¹⁷ was employed for paired comparisons

of the survival curves. For the mosquitoes released inside the cage, there was statistically significant evidence that Set I survival patterns were superior to Set II survival patterns ($P < 0.005$). However, the within-set comparisons were judged not to be different for Set I and Set II, so the data were pooled. As a result, average percent survival was plotted on semilog paper against time in days. The linear trend in the graph for Set I suggested a constant rate of mortality with age (Fig. 3).

TABLE 2

Survival of *Cx. pipiens* in Decator Cage at Fort Washington, Md, from 14 December 1978 to 8 March 1979

Accumulative time in days	Set I					Set II				
	Wild- caught	Lab-reared diapausing		Average percent survival (lx)	Average mortality rate/ 100/day	Lab-reared			Average percent survival (lx)	Average mortality rate/ 100/day
		Nonblood- fed	Blood-fed non-gravid			Diapausing	Nondiapausing			
						Blood-fed gravid	Nonblood- fed	Blood-fed		
0*	69	130	44	100		43	94	86	100	
4	68	127	42	97.5	0.62	37	91	85	95.5	1.12
11	65	124	42	95.1	0.36	35	84	85	91.5	0.6
18	65	116	38	90.1	0.74	26	76	79	81.2	1.61
25	63	110	37	86.4	0.59	24	67	71	72.6	1.50
32	61	108	37	84.8	0.27	20	57	64	63.2	1.85
39	60	104	37	82.7	0.35	20	45	51	52.0	2.53
46	57	97	35	77.8	0.85	14	35	38	39.0	3.57
53	56	93	34	75.3	0.45	10	22	25	25.6	4.93
60	55	88	34	72.8	0.47	6	15	13	15.2	5.76
74	45	68	28	58.0	1.45	4	9	5	8.1	3.36
84†	40	59	25	51.0	1.20	3	2	0	2.2	7.22

* 14 December 1978.

† 8 March 1979.

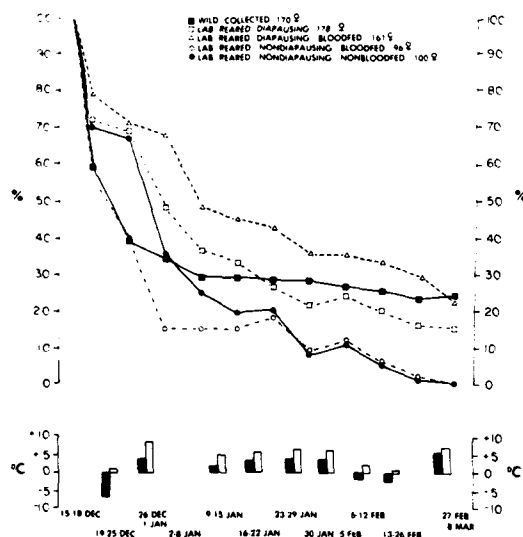


FIGURE 1. Percent survival of physiologic groups of *Culex pipiens* in room of Decator bunker, Fort Washington, Md., 14 December 1978 to 8 March 1979. (■) Average low temperature during period. (□) Average high temperature during period.

Whereas, the convex curve exhibited by Set II indicated that mortality rates were age-dependent (i.e., mortality rates steadily increased with age of the mosquitoes).

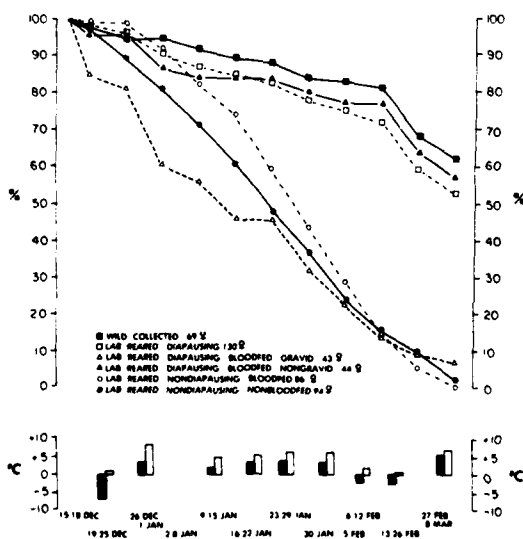


FIGURE 2. Percent survival of physiologic groups of *Culex pipiens* in cage at Decator bunker, Fort Washington, Md., from 14 December 1978 to 8 March 1979. (■) Average low temperature during period. (□) Average high temperature during period.

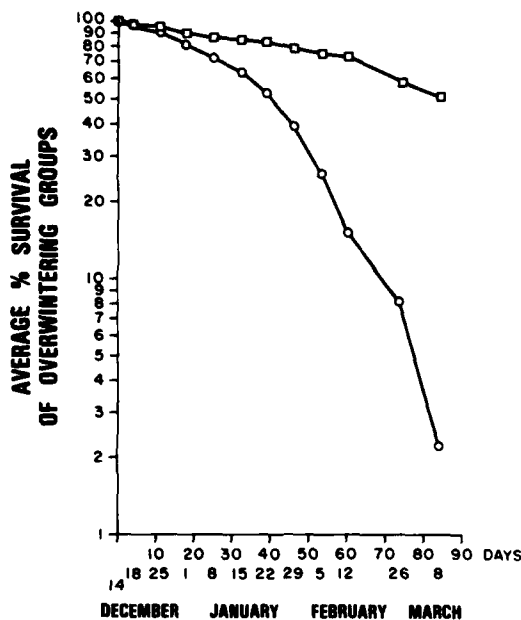


FIGURE 3. Average percent survival comparisons between Set I (□; wild caught, lab-reared diapausing blood-fed nongravid and lab-reared diapausing nonblood-fed) and Set II (○; lab-reared diapausing blood-fed gravid, lab-reared nondiapausing blood-fed and lab-reared nondiapausing nonblood-fed).

Weibull Model

It was found that both of these latter curves could be approximated by the "Weibull Model":¹⁸

$$N(t) = N_m e^{-\alpha t^\beta}$$

where $N(t)$ = number of females surviving at time t ; N_m = initial population entering diapause; e = natural logarithm, approximately 2.7183; t = time in days and; α , β = empirically derived constants. We first attempted to fit the model to the curves of Set I and II by taking the logarithm of minus the logarithm of both sides of the equation. Estimation of the parameters α and β followed the ordinary fitting of the resulting straight-line model. However, a better fit (Fig. 4) was produced using the nonlinear regression program P3R from the BMD series.¹⁹ Bradley has demonstrated that this approach yields maximum likelihood estimates in the exponential family.²⁰

DISCUSSION

Comparing the first two graphs, several differences are observed between survivorship in the

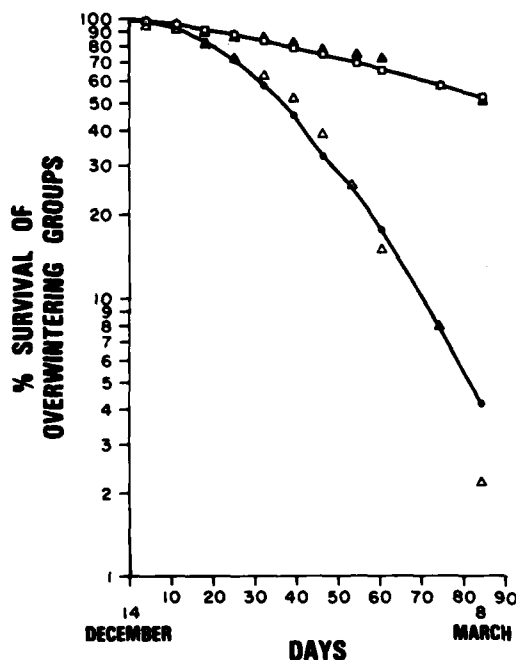


FIGURE 4. Comparison of curves of maximum likelihood estimation (MLE) for Set I (wild-caught, lab-reared diapausing blood-fed nongravid and lab-reared diapausing nonblood-fed) and Set II (lab-reared diapausing blood-fed gravid, lab-reared nondiapausing blood-fed and lab-reared nondiapausing nonblood-fed) using Weibull model, $N_{(t)} = N_{(0)} e^{-\alpha t^\beta}$, where $N_{(t)}$ = number of females surviving at time t ; $N_{(0)}$ = initial population entering diapause; e = natural logarithm, approximately 2.7183; t = time in days; α, β = empirically derived constants. (□) MLE for Set I, $\alpha = 0.0018$, $\beta = 1.33$. (●) average percent survival of Set I. (▲) MLE for Set II, $\alpha = 0.00096$, $\beta = 1.83$. (△) average percent survival of Set II.

cage versus that in the room. One explanation for the rapid initial decrease in the room might be the dispersal of females from the room by some unknown means shortly after release, which would alter the "overall survival" at the termination of the experiment. Evidence suggests that this was a factor, as a few marked mosquitoes were collected in adjoining rooms. Although not studied, predation pressure may also have been important in influencing survival rates of the females free in the room as numerous spiders and their webs were seen on the walls and ceiling throughout the study period. High RH is critical to the survival of adult mosquitoes. Unfortunately, RH was monitored only in the room and not inside the plexiglass cage, thus the possible influence, if any, of RH on the

survivorship in these two environments cannot be ascertained. Because of these uncontrolled parameters, it is impossible to draw any comparisons regarding the shapes of the survivorship curves for the females in the room relative to those curves for the cage.

Disregarding the shapes of the curves, for the mosquitoes in the room the numbers of females in each group surviving at the termination of the experiment indicate that the physiologic state of the female at the time of entering a hibernaculum in the fall is crucial to overwintering survival. Those females that had been preconditioned for diapause survived the winter in statistically the same proportions as the overwintering females naturally occurring in the bunkers at Fort Washington, regardless of whether the females had taken blood meals prior to release. The blood-fed mosquitoes, therefore, had an equal chance of surviving the winter as did the nonblood-fed and wild-caught mosquitoes. The survivorship of the blood-fed diapausing group was somewhat higher than either of the latter two groups until the last count on 8 March, although this difference was not significant.

Inside the cage there was a dramatic difference between the survival rates of the females in the groups of Set I compared to those in Set II (Fig. 2). At the termination of the experiment the average percent survival of Set I was 51.0% versus 2.2% for Set II. Since it was possible to recover females that died, ovarian development could be determined in those that had been previously blood-fed and failed to survive. Of particular interest is that the lab-reared diapausing blood-fed females could be separated into two groups depending on whether the females became gravid or remained nongravid. The nongravid group survived the winter at statistically the same rate as the wild-collected group and the lab-reared diapausing nonblood-fed group, whereas those that became gravid failed to survive. The mortality rate of this latter group was very similar to that observed in both of the nondiapausing groups. We believe that those females which became gravid upon blood-feeding were not in diapause, even though they had been environmentally preconditioned. That is, it appears that when a population of larvae and pupae of *Cx. pipiens* are being preconditioned for diapause, a certain proportion of the individuals do not physiologically respond to the environmental stimuli and, therefore, do not enter diapause as adults. It is mosquitoes in this

latter group that develop eggs when given a blood meal. We do not know if a proportion of the natural population in the late summer and fall behave similarly and do not enter diapause after preconditioning. However, if this is true, this phenomenon may have important evolutionary significance concerning overwintering survival, particularly in regions where moderately mild fall climates prevail. In these latter areas where fall temperatures are periodically mild, permitting adult mosquito activity and development of immatures, those females not physiologically in diapause may take a blood meal, develop eggs and produce another generation of adults that will further augment the number of females in the overwintering population. It is possible that natural selection has acted in areas such as coastal Maryland and New Jersey to maintain genetic polymorphism regarding diapause that affects the adult female's response to environmental stimuli.

Of epidemiological importance are those females that take a blood meal when exposed to warm temperatures, but fail to develop eggs and have a high overwintering survival rate. We believe that our study provides evidence that overwintering blood-fed females could serve as reservoirs for SLE virus. Females have been found naturally infected, they take blood meals under laboratory conditions of warm temperature that do not result in egg development, and they are capable of surviving in the same proportions as nonblood-fed diapausing females. The question is, what evidence is there that a proportion of females of *Cx. pipiens* in nature actually take a blood meal during diapause? Unfortunately, there is no reliable method for determining if a female has taken a previous blood meal which did not result in egg production. Therefore, in order to postulate that blood-feeding occurs, blood-engorged females must be collected during the late fall when essentially all reproductive activity has ceased. On 1 November 1979 we collected such a blood-engorged female of *Cx. pipiens*, along with four unengorged specimens, in a wooden shelter at a field study site on the Eastern shore of Maryland. These females were returned to our laboratory and placed in a climatic chamber without oviposition substrate at 15°C and 9:15 hours L:D cycle. After 2 weeks the blood-fed female was dissected; she displayed no evidence of parity or ovarian development. The female had a hypertrophic fat body indicative of a female in the state of diapause. For the first time this provides direct evidence that such blood-feeding does occur in nature in diapausing females.

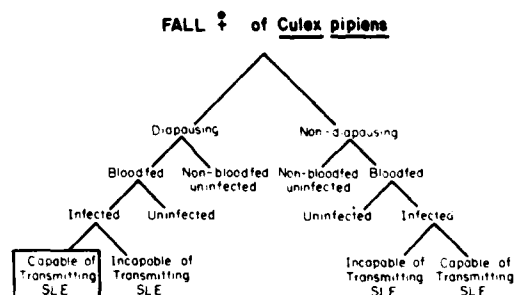


FIGURE 5. Hypothetical diagram of fall population of *Culex pipiens* females.

For the mosquitoes inside the cage the curve for Set I was almost linear, when the average percentages were plotted on semilog paper versus time, indicating that the rate of mortality for these diapausing females is almost constant and independent of the age of the individuals in the groups (Fig. 3). With the exception of the last two intervals, the mortality rate per 100 per day varied between 0.27 and 0.85 with a mean of 0.52 ± 0.19 . The increased mortality rates (1.45 and 1.20) for the last two periods may be due to the 14 days of below freezing temperatures between 12 and 26 February. The probability of a single diapausing female dying or surviving at the beginning of the experiment in December was about the same as that in the middle of February. The probability of survival for the nondiapausing groups and the "diapausing" blood-fed gravid group predominantly decreased from 12 December to 12 February, even disregarding the final two periods of the experiment. The shape of this latter survivorship curve closely approximates previous survivorship curves and age-specific mortality rates reported for *Culex quinquefasciatus*^{21, 22} and *Cx. tarsalis*.¹⁶ Nothing is known about the impact on survival of predation, climate and other ecological factors that would need to be taken into account in constructing a more generalized model; however, the close fit of the curves generated using the Weibull model with the observed survivorship curves of Sets I and II in the cage (Fig. 4) suggest a starting point for predicting survival rates and overall survivorship of an overwintering population of *Cx. pipiens*. For those latter populations, given the values of α and β , it is possible to predict the number of overwintering females at any time t (from t_0 to t_n). Many factors need to be addressed before construction of a generalized model is possible, including a comparison of the survival fitness of the SLE-infected females with that of the

noninfected females. Notwithstanding vertical transmission, in order to develop this type of model regarding the overwintering of SLE virus, it is necessary to have some idea of the proportion of diapausing females entering hibernacula that had taken infective blood meals and were capable of later transmitting the virus (Fig. 5).

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